RESPONSE

I. Status of the Claims

Prior to the fourth Action, claims 1, 14, 18, 23, 51, 52, 94, 96-99, 106, 107, 112, 117 and 122-149 were pending and have been examined. Claims 107, 130, 131, 148 and 149 are allowed; and claims 134, 138 and 144 are allowable. Presently, claims 1, 94, 96, 97 and 99 have been amended, without prejudice or disclaimer. Claims 98, 107, 132-134, 136-138, 142-144, 148 and 149 have been cancelled, without prejudice or disclaimer. No claims have been added.

Claims 1, 14, 18, 23, 51, 52, 94, 96, 97, 99, 106, 112, 117, 122-131, 135, 139-141 and 145-147 are therefore in the case. According to 37 C.F.R. § 1.121(c), a copy of the pending claims is provided in the amendment section.

II. Support for the Claims

Support for the revised claims exists throughout the specification and claims of the original and parent applications and particularly in the pending claims.

Composition claims 1 and 94 have each been revised to define the antibody in terms of the two recited variable regions. This is supported throughout the specification as filed, and particularly by allowed claim 107.

Pharmaceutical claim 96 has been revised to define the antibody in terms of the two recited variable regions. This is supported throughout the specification as filed, and particularly by allowed claim 148.

Kit claim 97 has been revised to define the antibody in terms of the two recited variable regions. This is supported throughout the specification as filed, and particularly by allowed claim 149.

Finally, method claim 99 has been revised to define the antibody in terms of the two recited variable regions. This is supported throughout the specification as filed, and particularly by allowed claim 107.

It will therefore be understood that no new matter is included within any of the amended claims.

III. Rejection Under 35 U.S.C. § 112, First Paragraph as to Deposit

The fourth Action at pages 2-4 newly rejects claims 106, 112, 117 and 122-129 under 35 U.S.C. § 112, first paragraph, as lacking complete evidence of the deposit of biological materials, *i.e.*, the hybridoma producing the 3G4 antibody. The rejection is overcome.

The specification as filed lists the name and address of the depository, the depositor, the dates of submission, the dates of receipt, the viability, the ATCC accession number, that the deposit was made under the provisions of the Budapest Treaty and that the hybridoma "will be made available by the ATCC under the terms of the Budapest Treaty upon issue of a U.S. patent with pertinent claims".

As to the fourth Action's requirement for a declaration stating that the biological materials will be irrevocably and without restriction or condition released to the public upon the issuance of a patent, Applicants provide herewith the Declaration of Biological Culture Deposit by Philip E. Thorpe, where Dr. Thorpe makes the appropriate statements.

The first rejection under 35 U.S.C. § 112, first paragraph is therefore overcome and should be withdrawn.

IV. Rejection Under 35 U.S.C. § 112, First Paragraph as to Enablement

The fourth Action at pages 4-12 newly rejects claims 1, 14, 18, 23, 51, 52, 94, 96-99, 132, 133, 135-137, 139-143 and 145-147 under 35 U.S.C. § 112, first paragraph, as allegedly lacking enabling support in the specification. Although Applicants respectfully traverse, the rejection is overcome.

The fourth Action acknowledges enabling support for claims reciting three heavy chain CDRs **and** three light chain CDRs; and acknowledges enabling support for claims reciting either the heavy chain variable region **or** the light chain variable region (fourth Action throughout, *e.g.*, pages 4 and 5). However, the fourth Action alleges that enabling support is lacking for antibodies with two variable regions that each comprises three CDRs in which only three of the CDRs are specified by sequence (fourth Action throughout, *e.g.*, page 5). Although the fourth Action at pages 4-12 repeats general comments regarding antibodies and cites certain references, the Action does not provide any actual reasoning in support of the rejection, but rather itself evidences sufficient enablement. The cited references also fail to support the rejection.

As the fourth Action likely appreciates, claims reciting three heavy chain CDRs and three light chain CDRs, but not reciting any framework sequences at all, are enabled, for example, by techniques including humanization and CDR grafting, known in the art prior to the invention and described in the specification as filed. Claims reciting a particular variable region with three CDRs and the framework sequences, but not reciting any other CDRs or framework sequences at all, are enabled, for example, by techniques including phage display and chain shuffling, in which libraries are screened to select complimentary variable domains, also known in the art prior to the invention and described in the specification as filed. The fourth Action's indication of allowable subject matter acknowledges enabling support for these two groups of claims.

However, the fourth Action does not include any reasoning as to why the remaining claims lack enabling support, nor do any of the cited references support the rejection. Rather, the only position in the fourth Action, which is repeated using slightly different phrasing at pages 4-12, is that six CDRs and all supporting framework sequences are always necessary. However, such a position is contradicted by the two groups of allowable claims and the Action's own indication of enabled subject matter. Namely, that three CDRs and framework sequences are sufficient without any other CDRs or framework (fourth Action at top of page 5) and that no framework sequences need to be recited when six CDRs are present (fourth Action at bottom of page 4). Thus, a proper *prima facie* rejection has not been established.

Taking the fourth Action's reasoning itself, it is agreed that [rodent] CDRs can be grafted into [human] framework and function effectively. Thus, there is enabling support for preparing one chain in which three CDRs are defined by sequence. It is also agreed in the fourth Action that one chain can be kept constant and a completely new chain, without definition of any CDRs or framework, can be selected and function effectively. Thus, enabling support clearly exists for the rejected claims, as one need only perform two steps, each of which are acknowledged to be enabled. There is nothing in these techniques, which are agreed to be enabled, that would render them lacking in enablement when performed together (either grafting and screening, or two screening steps using phage display).

The fourth Action does not include any reasoning as to why two routine steps could not be performed in combination; for example, why one screening step using phage display is enabled, but two such steps are not enabled. Nor do any of the cited references raise any doubts about such techniques, either alone or in combination, or otherwise suggest that the rejected claims lack enabling support.

As to the cited references in particular, the fourth Action cites Paul (1993), Rudikoff (1982), Colman (1994), Bendig (1995), MacCallum (1996) and Casset (2003) as allegedly supporting the position that six CDRs and all supporting framework sequences are required. The fourth Action also cites Bevers (2004), Luster (2006) and Ran (2005) as allegedly supporting the position that the screening methods as contemplated in the specification would not permit selection of an antibody of the rejected claims. In fact, none of these references support the Action's positions, or otherwise suggest that it would require undue experimentation to obtain an antibody of the rejected claims in light of the present disclosure.

As to Paul (1993), Rudikoff (1982), Colman (1994), Bendig (1995), MacCallum (1996) and Casset (2003), it is first noted that many of these references were published considerably before Applicants' priority date, with Rudikoff, for example, being published 20 years before the priority date, and now 26 years ago. Thus, many of the cited references are out-dated publications, which cannot be used to cast doubt on the enabling support in the specification or the level of skill in the art when the application was filed. In any event, even if these references were all contemporary, they either lack relevance to the rejected claims and/or fail to support the rejection (and are all contradicted by the Action itself, as set forth above).

For example, Rudikoff (1982) and Casset (2003) are *prima facie* irrelevant to the rejected claims. Rudikoff is cited for the position that changes in the amino acid sequence of CDRs may, in *some* situations, alter antigen-binding specificity (Rudikoff at abstract, emphasis as in original). MacCallum (1996) is later cited for a similar position, *i.e.*, that non-contacting residues within CDRs can define canonical backbone conformations. Casset is cited for the position that not just one CDR is essential for antigen binding and that CDRs other than CDR H3

play an important role (fourth Action at page 8). Even if correct, which is not the case[1], these issues are irrelevant as the rejected claims do not recite changes in the amino acid sequence of the CDRs and do not recite just one CDR. As to the rejection at hand, Rudikoff and Casset (and MacCallum) fail to support the rejection as they do not cast doubt on the ability of those of ordinary skill in the art to prepare an antibody recited in the claims, such as by performing two steps that are routine in the antibody field.

Paul (1993), Colman (1994) and MacCallum (1996) are cited for general antibody information, such as naturally-occurring antibodies, *i.e.*, those resulting from somatic recombination and hypermutation, use heavy and light chain variable regions to form antigen-binding sites (Paul), that conservative substitutions may abolish binding (Colman) and that certain residues outside the standard [Kabat] CDR definitions make antigen contacts (MacCallum)[2]. Aside from the fact that information in these references supporting Applicants' position has been overlooked[3,4], Paul, Colman, MacCallum (and Bendig[2]) do not support the rejection as they fail to cast doubt on known techniques available to practice the invention of the rejected claims as of their own publication dates, let alone as of the priority date of the present application.

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[1]Casset more supports the use of just one CDR, rather than argues against it; as do other published references, including Qiu *et al.*, *Nature Biotechnology*, 25(8):921-929, 2007 and Kiss *et al.*, *Nucleic Acids Research*, 34(19):e132, 2006, each made of record in Applicants' last response.

[2]Bendig shows that, as of 1995, reliable methods existed for humanizing rodent antibodies by grafting their CDRs into human antibodies. Bendig does not show that all six CDRs are required for antigen binding, which is anyway contradicted by the current allowance of claims including only three CDRs.

[3]For example, Colman states that a non-conservative substitution may have very little effect on binding affinity (fourth Action at page 7; Colman at page 35) and supports enablement by teaching that amino acid changes are tolerated, even at the antibody-antigen interface, and that antibodies have conformational adaptability in antigen binding (Colman throughout, *e.g.*, page 33, left column; page 35, right column).

[4]Although MacCallum states that certain residues outside the standard [Kabat] CDR definitions make antigen contacts, this only applies in between 1 and 6 instances from 26 cases (MacCallum at page 733, column 2). The Action itself anyway acknowledges enablement for claims that do not recite any residues outside the standard CDR definitions.

As to Bevers (2004), Luster (2006) and Ran (2005), these references do not cast doubt on the screening methods of the specification (fourth Action at page 11) because, as known by those of ordinary skill in the art and taught in the specification, one need only perform such screening methods in the presence of serum, as performed throughout the working examples of the specification, including Example IV. Again, as throughout the rejection, the Action's position is anyway contradicted by its own reasoning, as screening methods to select an entirely new variable chain are clearly enabled.

For the foregoing reasons, it is evident that a proper *prima facie* rejection has not been established. In particular, to support this rejection, the Office would need to provide reasoning, typically supported by references, showing that two admittedly enabled steps, routine in the antibody field, could not be performed in order to arrive at the invention of the rejected claims. Neither the fourth Action itself, nor any of the cited references, even approaches the required standard. The burden has thus been improperly shifted to the Applicants. *In re Wright*, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). Nonetheless, further evidence of enabling support is apparent in the art.

If fact, the art shows enabling support for the use of phage display, as disclosed in the present specification, to obtain antibodies in which only one or two CDRs are maintained, let alone three CDRs, as recited in the rejected claims. By way of example only, Rader *et al.*, *Proc. Natl. Acad. Sci. USA*, 95:8910-8915, 1998 **(Exhibit A)** shows, as of 1998, the successful use of phage display to select humanized antibodies containing only two rodent CDRs. Klimka *et al.*, *Brit. J. Cancer*, 83:252-260, 2000 **(Exhibit B)** evidences enabling support for the use of phage display to successfully generate a substantially human antibody containing only one murine CDR.

Neither the fourth Action, nor any of the cited references, offers anything to counteract the successes exemplified by the Rader and Klimka publications and the rejection is therefore improper. The issue of conducting two screening steps, rather than one, is not a proper basis for an enablement rejection, as it has long been held that the need for some experimentation does not render a claimed invention unpatentable under 35 U.S.C. §112. Indeed, a considerable amount of experimentation may be permissible if such experimentation is routinely practiced in the art. *In re Wands*, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988); *In re Angstadt and Griffin*, 190 USPQ 214 (CCPA 1976). See also, *United States vs. Telectronics, Inc.*, 8 USPQ2d 1217 (Fed. Cir. 1988), confirming that time and expense fail to show undue experimentation.

Therefore, the rejection was improper and is overcome. Nonetheless, and without acquiescing with the present rejection in any way, Applicants elect to place this application in condition for issue using the allowed and allowable claims[5].

The second rejection under 35 U.S.C. § 112, first paragraph is therefore overcome and should be withdrawn.

V. Enclosed Reference

The fourth Action enclosed a copy of Co & Queen, "Humanized Antibodies for Therapy", *Nature*, 351:501-502, 1991, but did not cite this reference in the body of the Action or list it on the PTO-892. Applicants therefore request clarification from the Office in the next communication as to whether or not the enclosed Co & Queen reference was intended as part of a rejection and so should be listed on a PTO-892.

[5]Composition claims of broader scope than those rejected have already issued as U.S. Patent No. 7,247,303 (Attorney Docket No. 4001.003085) and other claims drawn to combinations with second therapeutic agents can be pursued in co-pending application Serial No. 10/642,116 (Attorney Docket No. 4001.003087).

VI. Conclusion

This is a complete response to the referenced Official Action. In conclusion, Applicants submit that, in light of the foregoing remarks and accompanying documents, the present application is in condition for allowance and such action is respectfully requested. Should Examiner Goddard have any questions or comments, a telephone call to the undersigned Applicants' representative is earnestly solicited.

Respectfully submitted,

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EXHIBIT A

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Medical Sciences

A phage display approach for rapid antibody humanization: Designed combinatorial V gene libraries

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ABSTRACT **The development of a new strategy for antibody humanization is described. This strategy incorporates key recognition sequences from the parental rodent antibody into a phage display-based selection strategy. The original sequences of the third complementarity-determining regions (CDRs) of heavy and light chains, HCDR3 and LCDR3, were maintained and all other sequences were replaced by human sequences selected from phage-displayed antibody libraries. This approach was applied to the humanization of mouse mAb LM609 that is directed to human integrin $\alpha_v\beta_3$ and has potential applicability in cancer therapy as an antiangiogenic agent. We demonstrate this approach (*i*) provides a rapid route for antibody humanization constraining the content of original mouse sequences in the final antibodies to the most hypervariable of the CDRs; (*ii*) generates several humanized versions with different sequences at the same time; (*iii*) results in affinities as high as or higher than the affinity of the original antibody; and (*iv*) retains the antigen and epitope specificity of the original antibody. The production of multiple humanized variants may present advantages in the selection of antibodies that are more readily expressed on a large scale and could be important in therapeutic regimens that call for long-term treatment with antibodies in which antiidiotypic responses might be avoided by administration of alternative antibodies.**

Since the development of the hybridoma approach (1), a large number of rodent mAbs with specificity for antigens of therapeutic interest have been generated and characterized. The fact that rodent antibodies are highly immunogenic in humans, however, severely limits their clinical applications, especially when repeated administration is required for therapy. As a means of circumventing this limitation, several strategies have been developed to convert rodent antibody sequences into human antibody sequences, a process termed antibody humanization. Ideally, antibody humanization must not diminish specificity and affinity toward the antigen whereas immunogenicity must be completely eliminated. It has become apparent that the accomplishment of both aims is usually a time-consuming and costly undertaking with even the most current humanization strategies. Here, we report the development of a new humanization strategy that combines rational design with combinatorial selections using phage display. We demonstrate that this approach provides a rapid route to antibody humanization and demonstrate its application to the humanization of mouse mAb LM609 which is directed against the human integrin $\alpha_v\beta_3$. We chose LM609 as a model antibody for our humanization strategy because of its clinical potential. Recent findings by Brooks *et al.* (2–4) in a chorioallantoic membrane model and a severe combined immunodeficient mouse/human skin chimeric model have shown that LM609, when administered i.v., is able to reduce growth and metastasis of human tumors due to the inhibition of angiogenesis induced by the tumors. These findings suggest that integrin $\alpha_v\beta_3$ may be a target and LM609 a tool for cancer therapy.

MATERIALS AND METHODS

Proteins and Cell Lines. Human integrin $\alpha_v\beta_3$ was purified from human placenta as described (5). Human integrin $\alpha_{IIb}\beta_3$ was purchased from Enzyme Research Laboratories (South Bend, IN). mAb LM609 was described previously (6) and mAb AP3 was kindly provided by P. Newman (Milwaukee Blood Center, Milwaukee, WI). LM609 Fab was generated from IgG by digestion with immobilized papain using the ImmunoPure Fab Preparation kit from Pierce and separated from Fc and undigested IgG by three consecutive runs on a protein A column. CS-1 hamster cells were transfected with either human β_3 or β_5 cDNA as described (7) and maintained in RPMI 1640 supplemented with 10% fetal calf serum and 500 μg/ml G-418 (Life Technologies, Gaithersburg, MD) at 37°C and in 7% CO_2.

cDNA Cloning of LM609. Total RNA was prepared from 10^8 LM609 hybridoma cells (6) using the RNA Isolation kit from Stratagene. Reverse transcription and PCR amplification of the Fd fragment- and light chain-coding sequences were performed essentially as described (8). Fd fragment- and light chain-coding PCR products were cut with *Xho*I/*Spe*I and *Sac*I/*Xba*I, respectively, and ligated sequentially into the appropriately digested phagemid vector pComb3H (9). The ligation products were introduced into *Escherichia coli* strain XL1-Blue by electrotransformation and subsequent steps were as described (10) to produce phage displaying Fab on their surface. Phage were selected by panning (10) against immobilized human integrin $\alpha_v\beta_3$. After two panning rounds, single clones were analyzed for LM609 Fab expression. Supernatants from cultures that had been induced by the addition of isopropyl β-D-thiogalactopyransoside (10) were tested for binding to $\alpha_v\beta_3$ by ELISA using goat anti-mouse $F(ab')_2$ conjugated to alkaline phosphatase (Pierce) as secondary antibody. The sequence of Fd fragment- and light chain-coding sequences of positive clones was determined by DNA sequencing.

Amplification of Human Light Chain and Fd Fragment Sequences. Total RNA was prepared from bone marrow of five healthy donors supplied by Poietic Technologies (Germantown, MD) shortly after aspiration using TRI REAGENT (Molecular Research Center, Cincinnati, OH) and was further purified by lithium chloride precipitation (11). First-strand cDNA was synthesized using the SUPERSCRIPT Preamplification System for First Strand cDNA Synthesis kit with oligo(dT) priming (Life Technologies). The generated five first-strand cDNAs were subjected to separate PCR amplifications. V_κ, V_λ, and V_H sequences of each of the first-strand cDNAs were amplified using the primers listed below. All amplifications were performed under standard PCR conditions using *Taq* polymerase (Pharmacia).

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Abbreviations: CDR, complementarity-determining region; FR, framework region.

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While the sense primers hybridize to sequences that encode the N-terminal amino acids of the various V_κ, V_λ, and V_H families, the antisense primers hybridize to sequences that encode the C-terminal amino acids of framework region 3 (FR3) of V_κ, V_λ, or V_H, respectively, which are highly conserved (12). The primers used for the amplification of human antibody sequences are V_κ sense primers: HSCK1-F, 5′-GGGCCCAGGCGGCCGAGCTCCAGATGACCCAGTCTCC-3′; HSCK24-F, 5′-GGGCCCAGGCGGCCGAGCTCGTGATGACYCAGTCTCC-3′; HSCK3-F, 5′-GGGCCCAGGCGGCCGAGCTCGTGWTGACRCAGTCTCC-3′; and HSCK5-F, 5′-GGGCCCAGGCGGCCGAGCTCACACTCACGCAGTCTCC-3′; V_κ antisense primers: BKFR3UN, 5′-CAGTAATACACTGCAAAATCTTC-3′; BK2FR3UN and 5′-CAGTAATAAACCCCAACATCCTC-3′; V_λ sense primers: HSCLam1a, 5′-GGGCCCAGGCGGCCGAGCTCGTGBTGACGCAGCCGCCCTC-3′; HSCLam1b, 5′-GGGCCCAGGCGGCCGAGCTCGTGCTGACTCAGCCACCCTC-3′; HSCLam2, 5′-GGGCCCAGGCGGCCGAGCTCGCCCTGACTCAGCCTCCCTCCGT-3′; HSCLam3, 5′-GGGCCCAGGCGGCCGAGCTCGAGCTGACTCAGCCACCCTCAGTGTC-3′; HSCLam4, 5′-GGGCCCAGGCGGCCGAGCTCGTGCTGACTCAATCGCCCTC-3′; HSCLam6, 5′-GGGCCCAGGCGGCCGAGCTCATGCTGACTCAGCCCCACTC-3′; HSCLam70, 5′-GGGCCCAGGCGGCCGAGCTCGGGCAGACTCAGCAGCTCTC-3′; HSCLam78, 5′-GGGCCCAGGCGGCCGAGCTCGTGGTGACYCAGGAGCCMTC-3′; and HSCLam9, 5′-GGGCCCAGGCGGCCGAGCTCGTGCTGACTCAGCCACCTTC-3′; V_λ antisense primer: BLFR3UN, 5′-GCAGTAATAATCAGCCTCRTC-3′; V_H sense primers: HFVH1-F, 5′-GCTGCCCAACCAGCCATGGCCCAGGTGCAGCTGGTGCAGTCTGG-3′; HFVH2-F, 5′-GCTGCCCAACCAGCCATGGCCCAGATCACCTTGAAGGAGTCTGG-3′; HFVH35-F, 5′-GCTGCCCAACCAGCCATGGCCGAGGTGCAGCTGGTGSAGTCTGG-3′; and HFVH4-F, 5′-GCTGCCCAACCAGCCATGGCCCAGGTGCAGCTGCAGGAGTCGGG-3′; V_H antisense primer: BFR3UN, 5′-CGCACAGTAATACACGGCCGTGTC-3′.

Construction of a Chimeric Mouse/Human Fd Fragment by Fusing V_H of LM609 to Human C_H1. The phagemid vector pComb3H containing the LM609 Fab sequence was used as a template for amplification of the sequence encoding the N-terminal FR1 through FR3 fragment of the LM609 V_H by the PCR primer pair PELSEQ (5′-ACCTATTGCCTACGGCAGCCG-3′)/BFR3UN (5′-CGCACAGTAATACACGGCCGTGTC-3′). By overlap-extension PCR (13), the PELSEQ/BFR3UN product was fused to a PCR fragment encoding the HCDR3 of LM609, FR4 of V_H, and the entire C_H1 domain of the human anti-gp120 antibody b8 (14). This fragment was generated from the PCR primer pair CR501 (5′-GACACGGCCGTGTATTACTGTGCGCGTCATAACTACGGCAGTTTTGCTTACTGGGGCCAGGGAACCCTG-3′)/CR301 (5′-GAGGAGGAGGAGGAGACTAGTTTTGTCACAAGATTTGGGCTC-3′). FR4 of b8 was chosen because it is identical to FR4 of the LM609 V_H, with the exception of the C-terminal amino acid, which is A for LM609 and S for b8. The product of the overlap-extension PCR was cut with *Xho*I/*Spe*I, ligated into the appropriately digested phagemid vector pComb3H, cloned, and the correct sequence was confirmed by DNA sequencing.

Substitution of the LM609 Light Chain by a Human Light Chain That Contains the LCDR3 of LM609. Using overlap-extension PCR, the amplified human sequences encoding the N-terminal FR1 through FR3 fragment of V_κ and V_λ were fused to PCR fragments encoding the LCDR3 of LM609 coupled to FR4 of human V_κ or V_λ and the human C_κ or C_λ domain. Two κ fragments were generated by the PCR primer pairs CR503 (5′-GAAGATTTTGCAGTGTATTACTGCCAACAGAGTAACAGCTGGCCTCACACGTTTGGCCAGGGGACCAAGCTG-3′)/T7B (5′-AATACGACTCACTATAGGGCG-3′) and CR508 (5′-GAGGATGTTGGGGTTTATTACTGCCAACAGAGTAACAGCTGGCCTCACACGTTTGGCCAGGGGACCAAGCTG-3′)/T7B using the sequence of the anti-gp120 antibody b11 in pComb3 as a template (14). FR4 of b11 was chosen because it is identical to FR4 of the LM609 V_κ, with the exception of the third amino acid, which is G in LM609 and Q in b11. The 23-bp overlap of CR503 with BKFR3UN and CR508 with BK2FR3UN allowed the fusion of the corresponding PCR products by overlap-extension PCR. A λ fragment was generated by the PCR primer pair CR510 (5′-GAYGAGGCTGATTATTACTGCCAACAGAGTAACAGCTGGCCTCACACGTTCGGCGGAGGGACCAAGCTG-3′)/CLext (5′-AGAGAGAGAGAGAGAGAGCGCCGTCTAGAATTATGAACATTCTGTAGG-3′) using CLext-primed, first-strand cDNA from human bone marrow as a template. The 21-bp overlap of CR510 with BLFR3UN allowed the fusion of the corresponding PCR products by overlap-extension PCR. The generated light chain-coding sequences were cut with *Sac*I/*Xba*I and ligated into the appropriately digested phagemid vector pComb3H that contained the chimeric mouse/human Fd fragment. Electrotransformation of the ligation products into *E. coli* strain ER 2537 (New England Biolabs) resulted in a light chain library consisting of 1.5×10^8 independent transformants. DNA sequencing revealed the correct assembly of the fused fragments. Four rounds of panning against immobilized human integrin $\alpha_V\beta_3$ were carried out using 200 ng of protein in 25 μl of metal buffer [25 mM Tris·HCl (pH 7.5), 137 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM $MnCl_2$] for coating, 0.05% Tween 20 in Tris-buffered saline for washing, and 10 mg/ml trypsin (Difco) in Tris-buffered saline for elution. Trypsinization was for 30 min at 37°C. The washing steps were increased from 5 in the first round to 10 in the second round and 15 in the third and fourth rounds. The output phage pool of each round was monitored by phage ELISA using sheep anti-M13 conjugated to horseradish peroxidase (Pharmacia) as secondary antibody. After the fourth round, phage were produced from single clones and tested for binding to $\alpha_V\beta_3$ by phage ELISA. Light chain-coding sequences of positive clones were analyzed by DNA sequencing using the primer OMPSEQ (5′-AAGACAGCTATCGCGATTGCAG-3′).

Substitution of the LM609 Fd Fragment by a Human Fd Fragment That Contains the HCDR3 of LM609. Three PCR fragments were fused in one step by overlap-extension PCR. Using the selected phagemids from the light chain panning as a template, fragment 1 was amplified with the PCR primer pair RSC-F (5′-GAGGAGGAGGAGGAGGAGGCGGGGCCCAGGCGGCCGAGCTC-3′)/lead-B (5′-GGCCATGGCTGGTTGGGCAGC-3′). While the sense primer RSC-F hybridizes to a sequence upstream of the light chain-coding sequence, the antisense primer lead-B hybridizes to a sequence upstream of the Fd fragment-coding sequence. The amplified human sequences encoding FR1 through FR3 of the V_H fragment (see above) were used as fragment 2. Fragment 3 was amplified with the PCR primer pair CR501/HIgG1-B (5′-GCAGAGCCCAAATCTTGTGACACTAGTGGCCAGGCCGGCCAG-3′) using the hybrid mouse/human Fd fragment (see above) as a template. The antisense primer HIgG1-B hybridizes to the 3′ end of the C_H1-coding sequence. Using the 21-bp overlap of lead-B with the HFVH-F primers and the 24-bp overlap of BFR3UN with CR501, the three fragments were fused and amplified with the PCR primer pair RSC-F/RSC-B (5′-GAGGAGGAGGAGGAGGAGCCTGGCCGGCCTGGCCACTAGTG-3′). The antisense primer RSC-B overlaps with HIgG1-B. RSC-F and RSC-B introduce two asymmetric *Sfi*I sites. To maintain high complexity, separate PCRs were performed for each selected phagemid from the light chain panning (fragment 1) and for each of the five V_H fragment pools derived from the five first-strand cDNA sources (fragment 2). The generated fragments encoding the selected human light chains linked to human Fd fragments

were cut with *Sfi*I and ligated into the appropriately digested phagemid vector pComb3H, generating a library of 3×10^7 independent transformants. DNA sequencing revealed the correct assembly of the fused DNA fragments. Four rounds of panning against immobilized human integrin $\alpha_V\beta_3$ were carried out as described for the light chain panning. The output phage pool of each round was monitored by phage ELISA. After the fourth round, soluble Fab was produced from single clones as described (10) and tested for binding to immobilized $\alpha_V\beta_3$ by ELISA using goat anti-human F(ab')$_2$ conjugated to alkaline phosphatase (Pierce) as secondary antibody. Light chain- and Fd fragment-coding sequences of positive clones were analyzed by DNA sequencing using the primers OMPSEQ and PELSEQ, respectively.

Flow Cytometry. Flow cytometry was performed using a FACScan instrument from Becton Dickinson. For each determination, 5×10^5 untransfected hamster CS-1 cells or hamster CS-1 cells transfected with either human β_3 or β_5 cDNA were analyzed. Indirect immunofluorescence staining was performed with 2 μg/ml Fab in 1% BSA, 25 mM Hepes, and 0.05% sodium azide in PBS supplemented with 1% nonimmune goat serum. A 1:100 dilution of fluorescein isothiocyanate-conjugated goat anti-human F(ab')$_2$ (Jackson ImmunoResearch Laboratories) was used for detection. Incubation with primary antibodies was for 1 h, with secondary antibodies for 30 min, at room temperature. Competition experiments were performed by adding a fourfold molar excess of LM609 or AP3 IgG to the incubation mixture with the primary antibodies.

Surface Plasmon Resonance. Association (k_{on}) and dissociation (k_{off}) rate constants for binding of mouse and humanized LM609 Fab to human integrin $\alpha_v\beta_3$ were determined by surface plasmon resonance on a Biacore instrument (Pharmacia). The sensor chip was activated for immobilization with *N*-hydroxysuccinimide and *N*-ethyl-*N'*-(3-diethyl aminopropyl)carbodiimide according to the methods outlined by Pharmacia. Human integrin $\alpha_V\beta_3$ was coupled to the surface by injection of 6–9 μl of a 50 ng/μl sample in 10 mM sodium acetate (pH 3.5). Between 5,000 and 10,000 resonance units were immobilized. Subsequently, the sensor chip was deactivated with 1 M ethanolamine (pH 8.5). Binding of Fab to $\alpha_V\beta_3$ was studied by injection of Fab in a range of concentrations (10–600 nM), using PBS as a running buffer. The sensor chip was regenerated with 10 mM HCl and remained active for at least 50 measurements. Based on five measurements at different Fab concentrations, the k_{on} and k_{off} values were calculated using Biacore kinetics evaluation software (Pharmacia) and the equilibrium dissociation constant, K_d, was calculated from k_{off}/k_{on}. The reliability of the data was validated by analyzing the binding of each Fab on at least two different sensor chips and by applying the internal consistency tests suggested by Schuck and Minton (15). In addition, the rough range of the K_d values was independently confirmed by competition ELISA using a procedure described by Friguet *et al.* (16).

RESULTS

cDNA Cloning of LM609. cDNAs encoding the Fd fragment and entire light chain were cloned by PCR from LM609-expressing hybridoma cells (6). The PCR products were cloned into the phage display vector pComb3H (9), which is derived from pComb3 (10), and engineered to facilitate the expression of Fab on the surface of M13 filamentous phage. Phage displaying LM609 Fab were selected by panning against immobilized human integrin $\alpha_V\beta_3$ and the corresponding cDNA sequences were determined. The cloned LM609 Fab purified from *E. coli* demonstrated specific binding to $\alpha_V\beta_3$ using the ELISA.

Humanization of the Light Chain of LM609. Our humanization strategy is outlined in Fig. 1. It involves two selection steps for the sequential humanization of the light chain and the Fd fragment of the heavy chain. Throughout these selections the only preserved sequences in the variable domains of light chain (V_L) and heavy chain (V_H) are two of six CDRs, LCDR3 and HCDR3.

For the humanization of the light chain, the mouse Fd fragment was substituted by a chimeric Fd fragment composed of mouse V_H linked to the human constant domain 1 of the heavy chain, C_H1. A single residue in the mouse FR4 was converted to the corresponding human residue, resulting in complete humanization of the FR4 region. Humanization of the light chain began by substituting the V_κ gene segment of LM609 by a human V_κ and V_λ gene library joined at the LCDR3 junction. As described for the Fd fragment, the FR4 region was humanized by one amino acid change and appended to a human C_κ region. To ensure a highly diverse V gene library, the human antibody sequences were amplified from cDNA prepared from the bone marrow of five healthy individuals using a variety of oligonucleotides that were designed to amplify most of the known human antibody sequences (see *Materials and Methods*). The corresponding phage libraries displaying hybrid Fab were combined and selected by four rounds of panning against immobilized human integrin $\alpha_V\beta_3$. Analysis of the output phage pool from each round for binding to $\alpha_V\beta_3$ by phage ELISA revealed an increasing signal. After the fourth round of selection, six clones that demonstrated strong reactivity to the antigen were studied. DNA sequence analysis of these clones revealed three different light chain sequences. Two light chains (Fig. 2*A*), found in five of six positive clones, differed in only four amino acids (i.e., they were 96% identical), whereas a third light chain sequence shared about 80% identity with the other two (data not shown). This latter sequence consisted of two parts, each of which could be aligned to germ-line genes from different V_κ families; thus, this light chain sequence probably arose from PCR crossover, which has been reported to occur frequently in the amplification of antibody sequences (17).

The selected human light chains are κ light chains as was the original mouse light chain. Databank screening revealed that the selected human light chains are derived from the same germ-line gene, namely, DPK-26, which belongs to the $V_\kappa 6$ family (Fig. 2*A*). This result supports a strong selection for the light chains since the $V_\kappa 6$ family represents only a small fraction of the expressed human V_κ repertoire (18, 19). An obvious reason for this strong selection is the strong sequence similarity between the selected human light chains and the original mouse light chain. Limited sequencing of the unselected library confirmed its diversity and did not reveal any $V_\kappa 6$-containing clones. Moreover, in contrast to the unselected sequences, both LCDR1 and LCDR2 of the selected human light chains are highly similar to the corresponding mouse sequence (Fig. 2*A*). The C-terminal amino acid of FR2 (Kabat position 49; ref. 12) of the original mouse light chain sequence is a lysine, which is an unusual amino acid at this position and, thus, may be involved in the formation of the antigen-binding site. Interestingly, this lysine is conserved in our selected sequences (Fig. 2*A*) as well as in the two known human

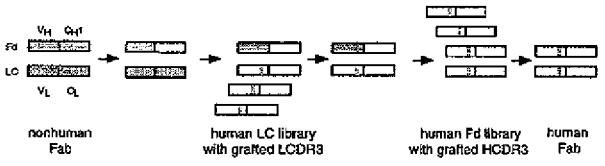

FIG. 1. Humanization of nonhuman monoclonal antibodies by a combination of CDR grafting and V gene shuffling. Nonhuman sequences are shown in gray, human sequences in white. In the first step, a chimeric nonhuman/human Fd fragment is used as a template for the selection of a human light chain that contains the grafted LCDR3 loop of the nonhuman light chain. In the second step, a human Fd fragment that contains the grafted HCDR3 loop of the nonhuman Fd fragment is selected. The sequential V gene shuffling procedure is based on phage display. LCDR3, complementarity-determining region 3 of light chain; HCDR3, complementarity-determining region 3 of heavy chain.

A V_L sequences

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
mouse	ELVMTQTPATLSVTPGDSVSLSC	RASQSISNHLH	WYQQKSHESPRLLIK	YASQSIS	GIPSRFSGSGSGTDFTLSINSVETEDFGMYFC	QQSNSWPHT	FGGGTKLEIK
DPK-26	-I-L--S-DFQ----KEK-TIT-	------GSS--	-----FDQ--K-----	-----F-	-V----------T---L-A--AAY-Y-		
human[1]	------S-EFQ----KET-TIT-	----D-GTS--	-----PGQ--K-----	----PVP	-V----R----------T-Y-L-A---AV-Y-	---------	--Q-------
human[2]	------S-EFQ----KET-TIT-	----D-G-S--	-----PGQ--K-----	----PVP	-V----R----------T-SRL-P---AV-Y-	---------	--Q-------

B V_H sequences

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
mouse	EVQLEESGGGLVKPGGSLKLSCAASGFAFS	SYDMS	WVRQTPEKRLEWVA	KVSSGGGSTYYLDTVQG	RFTISRDNAKNTLYLQMSSLNSEDTAMYYCAR	HNYGSFAY	WGQGTLVTVSA
human[3]	Q---VQ--AEVR---S-VRV--K---GT--	GFAV--	----A-GQ-P--LG	GIVASL---D-AQKF-D	KL--TV-ESTA-VYME-RN-R-D---V------	--------	----------S
human[4]	Q---Q---P-----SQI-S-T-TV--ASI-	RGGYYWS	-I--Y-GKG---IG	YIH HS------NPSLKS	-V--AI-TS--Q-S-KLT-VTAA---V------	--------	----------S
human[5]	Q---Q---P-----SQF-F-T-TV--GSI-	SGGYYWS	-I-HH-GKG---IG	YIH HRAAP--NPSLKS	-V---V-TSR-QIS-KLR-VTAA---V------	--------	----------S
human[6]	Q---Q---P-----SET-S-T-TV--GST-	SGGYYWS	-I--H-GKG---IG	YIH HSAG----NPSLKS	-V-M-V-TS--Q-S-KLT-VTAA---V------	--------	----------S
human[7]	Q---Q---P-----SET-S-T-SV--GSI-	SGGYYWS	-I-HH-GKG---IG	YIH HSAG---NPSLKS	-V-M-A-TS--Q-S-RLA-VTAA---V------	--------	----------S

FIG. 2. Amino acid sequence alignment of mouse and humanized LM609. Shown are FRs and CDRs. Dashes indicate identical amino acids. Note that due to our grafting procedure CDR3 is identical in the original mouse and the selected human sequences. (*A*) Alignment of the selected human V_L sequences. Databank screening revealed that the two selected human V_L sequences are derived from germ-line DPK-26 of the $V_\kappa 3$ family. (*B*) Alignment of the selected human V_H sequences were selected. Four of them, represented by clones 7, 4, 24, and 2, are highly related in amino acid sequence; they share an identical V_L domain and an amino acid sequence identity of at least 85% in their V_H domains. The V_H sequences are all derived from germ-line DP-65 or the highly related DP-78. In contrast, clone 11 represents a humanized version with a V_H domain that is derived from a different germ-line family. This humanized version also contains a different V_L domain which is 96% identical and derived from the same germ-line. Germ-lines were determined by nucleic acid sequence alignment using DNAPLOT software provided by the VBASE Directory of Human V Gene Sequences (http://www.mrc-cpe.cam.ac.uk/imt-doc/). [1]clone 11, germ-line DPK-26 ($V_\kappa 6$ family); [2]clones 2, 4, 7, 24, germ-line DPK-26 ($V_\kappa 6$ family); [3]clone 11, germ-line DP-10 ($V_H 1$ family); [4]clone 7, germ-line DP-78 ($V_H 4$ family); [5]clone 4, germ-line DP-65 ($V_H 4$ family); [6]clone 24, germ-line DP-65 ($V_H 4$ family); [7]clone 2, germ-line DP-65 ($V_H 4$ family).

germ-line $V_\kappa 6$ sequences, whereas all of the unselected sequences that were analyzed contained a tyrosine instead. The $V_\kappa 6$ family is the only human V_κ family that contains a lysine at this position. To study whether the selected human light chains are derived from germ-line V genes that are most similar to the original mouse light chain, the VBASE directory of human V gene sequences (maintained by I. M. Tomlinson, @ http://www.mrc-cpe.cam.ac.uk/imt-doc/) was searched for the highest sequence similarity with the original V_L. Indeed, germ-lines DPK-26 and DPK-25, the only two members of the $V_\kappa 6$ family, were determined to be most similar to the original mouse light chain. Thus, library and databank screening yielded the same result.

Three clones from the light chain selection demonstrated weaker binding to $\alpha_V\beta_3$ than the six clones discussed above, but still gave significant binding above background. DNA sequencing revealed three unrelated V_λ sequences that had no apparent similarity to the original mouse V_κ sequence. The V_λ sequences, along with the selected V_κ sequences (and with the exception of the clone containing the PCR crossover), were used as templates in the humanization of the heavy chain of LM609.

Humanization of the Heavy Chain of LM609. A library of Fd fragments was prepared by stitching human V_H gene libraries onto the chimeric Fd fragment described above. These libraries were paired with the four selected human light chains and were selected by four rounds of panning against immobilized human integrin $\alpha_V\beta_3$. As seen for the human light chain selection, analysis of the output phage pool from each round for binding to $\alpha_V\beta_3$ by phage ELISA revealed an increasing signal. After the fourth round, Fab was produced from single clones and tested for binding to $\alpha_V\beta_3$ by ELISA. Light chain- and Fd fragment-coding sequences from 14 binding clones were determined by DNA sequencing and revealed five different sequences (Fig. 2). The two human κ light chains with the highest sequence homology to the LM609 light chain were reselected. One of them was found to pair with four different Fd fragments that were closely related to each other (85–96% sequence identity) and derived from germ-line DP-65 or DP-78 of the $V_H 4$ family. The other human κ light chain was found to pair with a Fd fragment that was derived from germ-line DP-10 of the $V_H 1$ family. Neither germ-lines from the $V_H 4$ nor the $V_H 1$ family show high sequence homology with the V_H of LM609. Indeed, databank screening yielded a germ-line from the $V_H 3$ family as the best human match for the V_H of LM609. Phylogenetic analysis has shown that $V_H 1$, $V_H 3$, and $V_H 4$ not only form separate families but belong to different clans of the human V_H germ-lines (20). In retrospect, V_λ selections should have been performed independent of V_κ selections to ensure the retention of highly diverse light chains in the humanized antibodies. The V_λ antibodies may have been lost in the selections for reasons other than antibody affinity, such as their relative toxicity to *E. coli*.

Binding Specificity and Affinity of Humanized LM609. Five humanized LM609 versions, represented by clones 11, 7, 4, 24, and 2 were produced as soluble Fab by *E. coli*, purified by affinity chromatography, and their binding specificity and affinity was analyzed. Humanized LM609, which had been selected by binding to immobilized and thus potentially denatured human integrin $\alpha_v\beta_3$, was tested for binding to native human integrin $\alpha_v\beta_3$ expressed on the cell surface. For this, binding of humanized LM609 to untransfected CS-1 hamster cells and CS-1 hamster cells transfected with either human β_3 or β_5 cDNA (7) was analyzed by flow cytometry. By recruiting the endogenous hamster α_v polypeptide, the human β_3 and β_5 polypeptides form functional integrins on the cell surface (7). Like mouse LM609, and in contrast to unrelated human Fab fragments that were used as controls (data not shown), all five humanized versions of LM609 revealed specific binding to CS-1 hamster cells transfected with human β_3 (Fig. 3*A*). Binding of all five humanized LM609 versions to CS-1 cells transfected with human β_3 cDNA could be blocked by an excess of LM609. As shown in Fig. 3*B*, a fourfold molar excess of mouse LM609 IgG blocked binding of humanized LM609 almost completely, whereas the same concentration of mouse

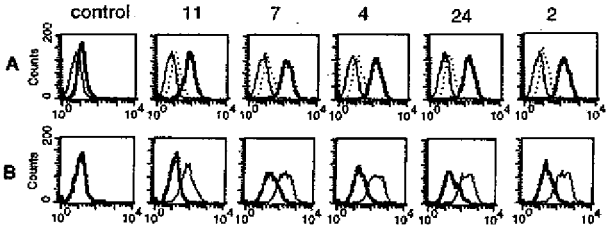

FIG. 3. Flow cytometry histograms demonstrating that humanized LM609 binds specifically to human integrin $\alpha_v\beta_3$ and can be blocked by mouse LM609. (*A*) Binding of humanized LM609 Fab to untransfected CS-1 hamster cells (fine line) and CS-1 hamster cells transfected with either human β_3 (bold line) or β_5 cDNA (dotted line). (*B*) Binding of humanized LM609 Fab to CS-1 hamster cells transfected with human β_3 cDNA in the presence of a fourfold molar excess of mouse AP3 IgG (fine line) or mouse LM609 IgG (bold line). Clones 11, 7, 4, 24, and 2 represent the five humanized LM609 versions (cf. Fig. 2). Controls in *A* and *B* are based on identical experiments using buffer instead of humanized LM609 Fab. The *y* axis gives the number of events in linear scale, the *x* axis the fluorescence intensity in logarithmic scale.

Table 1. Binding kinetics of mouse LM609 and humanized LM609 Fab

Clone	$k_{on}/10^4$, $M^{-1}\,s^{-1}$	$k_{off}/10^{-4}$, s^{-1}	K_d, nM
LM609*	14	4.6	3.3
LM609	8.6	8.6	10
11	1.0	16	160
7	18	5.4	3.0
4	6.8	5.8	8.5
24	13	9.9	7.6
2	11	7.5	6.8

Binding kinetics were determined using surface plasmon resonance. Human integrin $\alpha_v\beta_3$ was immobilized on the sensor chip. The K_d value was calculated from k_{off}/k_{on}. Clones 11, 7, 4, 24, and 2 represent the five humanized LM609 versions (cf. Fig. 2). Fab was produced by *E. coli* except LM609* which was prepared from IgG by papain digestion.

AP3 IgG directed to a different epitope on human integrin $\alpha_v\beta_3$ had no effect. A 20-fold molar excess of LM609 Fab derived from IgG by papain digestion also blocked the binding of humanized LM609 (data not shown). Control experiments revealed that both LM609 and AP3 bound to $\alpha_v\beta_3$ expressed on the cell surface. Potential cross-reactivity of humanized LM609 with human integrin $\alpha_{IIb}\beta_3$ was analyzed by ELISA. Whereas an engineered RGD peptide mimetic antibody Fab-9 with known cross-reactivity (21) bound to both immobilized human integrin $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$, cross-reactivity with $\alpha_{IIb}\beta_3$ was not detected for mouse LM609 nor its five humanized versions.

The kinetic parameters of Fab binding to human integrin $\alpha_v\beta_3$ binding were determined using surface plasmon resonance and the affinities were calculated from these kinetic parameters (Table 1). Analysis of these binding data revealed that the four humanized LM609 versions with the high sequence similarity, represented by clones 7, 4, 24, and 2, also show similar affinities for $\alpha_v\beta_3$. Clones 4, 24, and 2, which are derived from the same germ-lines, have almost identical K_d values in the range of 7–9 nM. Clone 7, which shares the same light chain but contains a Fd fragment that is derived from a different, though highly related V_H germ-line, has a moderately higher affinity with a K_d value of 3 nM. In contrast, clone 11 with the unrelated Fd fragment has a much weaker affinity, which is mainly caused by a lower association rate constant (Table 1). LM609 Fab was analyzed for comparison. As Table 1 shows, Fab that was generated by papain digestion from LM609 IgG revealed an affinity with a K_d value of about 3 nM, whereas the affinity of Fab produced by *E. coli* was weaker by a factor of three. It is likely that this discrepancy is partly due to a lower concentration of functional Fab in preparations from *E. coli*. However, the higher dissociation rate constant of *E. coli*-derived LM609 Fab, which is independent from the concentration, indicates that the quality of the antigen-binding site might be affected as well. In any case, the humanized LM609 versions were derived from *E. coli* as well and, thus, should be compared with the corresponding LM609 preparation. Such a comparison (Table 1) shows that four of five humanized antibodies have an affinity that is as good as or better than the original mouse antibody.

DISCUSSION

Though rodent mAbs have long been regarded as powerful therapeutic agents, a major obstacle for clinical applications has been their immunogenicity in humans. Two routes in antibody engineering have been taken to overcome the immunogenicity of mAbs, either the humanization of rodent mAbs or the direct generation of human mAbs. The latter route has recently gained importance with the development of new methodologies that allow the selection of human mAbs from immune, naive, and synthetic human antibody libraries displayed on phage (22, 23) as well as from transgenic mice (24). More than 20 yr of mAb generation by the classical hybridoma technology, however, has yielded a number of promising pharmaceutical candidates and their humanization compares well to the *de novo* generation and characterization of human mAb for accessing clinical applications in the coming years.

Currently, CDR grafting is the most frequently used strategy for the humanization of rodent mAbs (25). In this approach the six CDR loops comprising the antigen-binding site of the rodent mAb are grafted into corresponding human framework regions. CDR grafting takes advantage of the conserved structure of the variable Ig domains, with the four framework regions serving as a scaffold that supports the CDR loops. CDR grafting often yields humanized antibodies with much lower affinity because framework residues are involved in antigen binding, either indirectly, by supporting the conformation of the CDR loops, or directly, by contacting the antigen (26). Therefore, it is usually necessary to replace certain framework residues in addition to CDR grafting. The fact that about 30 framework residues potentially contribute to antigen binding (26) makes this fine-tuning step very laborious. Another humanization strategy is the method of resurfacing (27). In this approach only the surface residues of a rodent antibody are humanized.

Though both CDR grafting and resurfacing are based on rational design strategies and iterative optimization (i.e., site-directed mutagenesis of framework residues aided by computer modeling), selective approaches (i.e., randomization of a small set of framework residues and subsequent selection from phage display libraries) have been reported recently in humanization strategies (28, 29).

In vitro selection and evolution of antibodies derived from phage display libraries have become a powerful tool in antibody engineering (for recent reviews cf. refs. 9 and 30). An entirely selective humanization strategy based on phage display libraries has been reported by Jespers *et al.* (31). In two steps, each polypeptide of the rodent antibody, either light chain or heavy chain, is replaced by a corresponding human polypeptide library and the resulting hybrid antibody library is selected by panning against the particular antigen. Though this strategy may compete with CDR grafting because the arduous fine-tuning steps are unnecessary, the lack of other successful applications of this approach—and our failed attempts to humanize LM609 by this approach for comparative studies—suggest that in contrast to CDR grafting, the general applicability of this approach for antibody humanization is uncertain. Also, since this approach is a sequential chain shuffling procedure, it may lead to the production of a humanized antibody that recognizes a slightly different epitope (32–34). It has been observed that antibodies consisting of the same heavy chain paired with light chains that differ in LCDR3 and elsewhere in V_L may bind different epitopes on the same antigen. It is conceivable that this is an interesting feature in particular cases; however, alteration of antigen specificity following antibody humanization is not desired in general.

Based on these general considerations, we designed a strategy that recognizes the key roles of HCDR3 and LCDR3 in antigen recognition and combined this with a selective approach that eliminates the arduous fine-tuning steps associated with CDR grafting as well as all mouse sequence. This strategy for humanizing antibodies is presented in Fig. 1. In the first step a human light chain library with the grafted original LCDR3 replaces the original light chain and a chimeric Fd fragment consisting of the original V_H Ig domain fused to a human C_H1 Ig domain replaces the original Fd fragment. FR4 of both chains is directly humanized by simple point mutations prior to the first selective step. Sequence changes required in this region should in general be minimal given the substantial homology between mouse and human J genes and should have little effect on the affinity. Human constant regions are preferred to stabilize the hybrid Fab of the first selection step by the interaction of two matching human constant domains C_κ and C_H1. In addition, Fab carrying human constant regions are often better expressed in *E. coli* (35,

36; C.R. and C.F.B. III, unpublished observations), a prerequisite for phage display. In the second step, the selected human light chains from the first step are paired with a human Fd fragment library containing the original HCDR3. Since the heavy chain typically plays the most dominant role in antigen recognition, it is conserved in the first selection step.

By preserving the original LCDR3 and HCDR3 sequences of LM609 while subjecting the remaining sequence to selection, our humanization strategy was designed to ensure antigen specificity and epitope conservation. LCDR3 and HCDR3 contain the hypervariable joints of the V/J and V/D/J gene rearrangements that participate in direct antigen contact in all studied antigen/antibody complexes (37). Unlike the other CDR regions, both LCDR3 and HCDR3 interact with all three CDRs of the other variable domain (38). Thus, although generalizations might be misleading (37), LCDR3 and HCDR3 can be considered to make the most significant contributions to affinity and specificity. Given the tremendous sequence diversity displayed by human antibodies in these regions and the mechanism of its generation, it is difficult, if not impossible in most cases, to classify sequences of these regions as either mouse or human. Thus, from the perspective of sequence these antibodies may be considered completely human. Human HCDR3s are, however, on average longer than mouse HCDR3s and encompass the full range of lengths utilized by mice (39). The HCDR3 length of eight amino acids found in LM609 is well represented in both mouse and human antibodies (39). HCDR3 length will not likely be a significant issue in mouse to human conversions.

Antigen specificity and epitope conservation are critical demands in the humanization of LM609. LM609 binds to a conformational epitope on human integrin $\alpha_v\beta_3$. Importantly, by binding to this epitope LM609 induces apoptosis in vascular cells expressing $\alpha_v\beta_3$ (3). In contrast to antibodies that are engineered RGD peptide mimics (21), LM609 does not recognize the related human integrin $\alpha_{IIb}\beta_3$. A cross-reactivity with human integrin $\alpha_{IIb}\beta_3$, which is expressed on platelets, would preclude the use of LM609 as a tool in cancer therapy. The five humanized LM609 versions were analyzed for antigen specificity in terms of cross-reactivity with the human integrins $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_5$, which are closely related to $\alpha_V\beta_3$ in sequence and function (40). Neither $\alpha_{IIb}\beta_3$ nor $\alpha_V\beta_5$ was recognized by the five humanized versions of LM609. In addition, all appeared to bind to the same epitope on $\alpha_V\beta_3$ as LM609, as their binding was specifically blocked in the presence of a molar excess of LM609 but not of AP3, a mAb that binds a different epitope on human integrin $\alpha_v\beta_3$. In addition to the binding specificity, the binding affinity provides evidence that epitope conservation was obtained; three of five humanized LM609 versions bound $\alpha_v\beta_3$ with an affinity very similar to that of LM609. Yet, the contribution of the selected CDRs to the antigen-binding site is obvious from the fact that one humanized LM609 version binds $\alpha_v\beta_3$ with higher, and another with slightly lower, affinity.

The generation of different humanized versions of the parental antibody is an attractive result of this methodology. CDR grafting, in contrast, generates a single humanized version. It is anticipated that an antiangiogenic strategy for cancer therapy would require long-term administration of antibody. It is conceivable that repeated therapeutic application may produce an antiidiotypic response that ablates the efficacy of the antibody. Administration of an equally potent antibody that is unreactive to the antiidiotypic response generated by the first would allow therapy to continue. Indeed, introduction of modest changes within the variable domain of an antibody can dramatically alter its reactivity to an antiidiotypic response (41). Finally, a practical advantage of producing multiple humanized antibodies is that the expression level of antibodies is antibody dependent. Thus, one or more of the humanized antibodies may be more suitable for large-scale production.

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EXHIBIT B

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Human anti-CD30 recombinant antibodies by guided phage antibody selection using cell panning

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Summary In various clinical studies, Hodgkin's patients have been treated with anti-CD30 immunotherapeutic agents and have shown promising responses. One of the problems that appeared from these studies is the development of an immune response against the non-human therapeutics, which limits repeated administration and reduces efficacy. We have set out to make a recombinant, human anti-CD30 single-chain variable fragment (scFv) antibody, which may serve as a targeting moiety with reduced immunogenicity and more rapid tumour penetration in similar clinical applications. Rather than selecting a naive phage antibody library on recombinant CD30 antigen, we used guided selection of a murine antibody in combination with panning on the CD30-positive cell line L540. The murine monoclonal antibody Ki-4 was chosen as starting antibody, because it inhibits the shedding of the extracellular part of the CD30 antigen. This makes the antibody better suited for CD30-targeting than most other anti-CD30 antibodies. We have previously isolated the murine Ki-4 scFv by selecting a mini-library of hybridoma-derived phage scFv-antibodies via panning on L540 cells. Here, we report that phage display technology was successfully used to obtain a human Ki-4 scFv version by guided selection. The murine variable heavy (VH) and light (VL) chain genes of the Ki-4 scFv were sequentially replaced by human V gene repertoires, while retaining only the major determinant for epitope-specificity: the heavy-chain complementarity determining region 3 (CDR3) of murine Ki-4. After two rounds of chain shuffling and selection by panning on L540 cells, a fully human anti-CD30 scFv was selected. It competes with the parental monoclonal antibody Ki-4 for binding to CD30, inhibits the shedding of the extracellular part of the CD30 receptor from L540 cells and is thus a promising candidate for the generation of anti-CD30 immunotherapeutics. © 2000 Cancer Research Campaign

Keywords: CD30; phage display; chain shuffling; human antibody; guided selection

Although monoclonal antibodies (moab) raised by hybridoma technology (Köhler and Milstein, 1975) have been demonstrated to be very useful in research and diagnosis, they are somewhat problematic as binding moieties in immunotherapeutic agents for the treatment of tumours. Apart from their relatively large size (150 kDa), which makes it difficult to penetrate into solid tumours, these non-human antibodies generate an immune response resulting in serious side-effects such as serum sickness or anaphylactic shock, which prevent long-term treatment of cancer patients (Shawler et al, 1985). It is also documented that this human anti-mouse antibody (HAMA) response causes a rapid blood clearance of these reagents, which diminishes their efficacy (Khazaeli et al, 1994).

To circumvent these problems, two strategies have been followed. First, a reduction of the molecular size of the binding moiety using Fab fragments or even just the variable fragments of an antibody as a single-chain variable fragment (scFv) has significantly reduced the target surface for an immune response and thus the immunogenicity. Secondly, the use of humanized proteins like chimaeric or CDR-grafted, or even fully human antibodies or antibody fragments, has been demonstrated to reduce their immunogenicity (Meredith et al, 1993).

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Because of technical problems and difficulties in retrieving suitable human donors, it is complicated to raise human hybridomas by conventional techniques. However, the progress in molecular biology has offered different ways to evade this restriction. One possibility is the use of transgenic mice carrying human immunoglobulin genes. These mice can be used to generate hybridomas secreting human antibodies (Brüggemann and Neuberger, 1996). Another way is the use of human V-gene libraries expressed and displayed on phage and selection of antigen-specific antibodies therefrom (Hoogenboom, 1997; Winter et al, 1994). These libraries can be derived from immunized or non-immunized donors or even generated synthetically (Hoogenboom et al, 1998). Indeed, from very large phage libraries, high-affinity antibodies to many different target antigens can be selected (Hoogenboom, 1997). This *in vitro* selection procedure is subjected to a series of biases introduced by library preparation, selection conditions and the screening protocol. Strong biases in selected populations can arise, in particular when selecting on complex antigenic targets (Hoogenboom et al, 1999; Persic et al, 1999).

Therefore, it sometimes remains difficult to retrieve antibodies with desired properties like recognition of a unique epitope, induction of a post-binding signal transduction or internalization upon binding to a cell-surface receptor on the target cell (McCall et al, 1998). Hybridoma-derived antibodies with such characteristics are sometimes available, and may be converted to human versions using a method termed 'guided selection' (Jespers et al, 1994): by

two consecutive chain-shuffling procedures, the rodent antibody domains are swapped for human domains, using phage display technology (library construction and selection on antigen) to retrieve the best-matching partner. Our goal was to obtain a fully human antibody from the well-characterized murine moab Ki-4, which recognizes the CD30 receptor.

CD30 was originally identified by Schwab et al (1982) as the antigen abundantly expressed on Hodgkin–Reed Sternberg cells (H–RS) in primary Hodgkin's lymphoma and recognized by the first anti-CD30 moab Ki-1. Expression of CD30 in high copy numbers on the cell surface has also been reported for a subset of non-Hodgkin lymphomas (NHL), virally transformed B- and T-cell lines, a subform of large-cell anaplastic lymphoma ($CD30^+$-LCAL), embryonal carcinomas, malignant melanomas and mesenchymal tumours (Gruss and Dower, 1995). The CD30 receptor is therefore a useful clinical and pathological tumour marker for these diseases and a good target for immunotherapy.

Here we report the synthesis of a human anti-CD30 scFv (hAK30) on the basis of the murine anti-CD30 moab Ki-4. Murine moab Ki-4, which shows no detectable cross-reactivity with vital human organs, has successfully been used as part of a chemically linked ricin A immunotoxin in vivo (Schnell et al, 1995) and also as a scFv in a *Pseudomonas* exotoxin A-based recombinant immunotoxin in vitro (Klimka et al, 1999). Therefore, we exchanged the murine variable heavy (VH) and light (VL) chain genes with human counterparts with respective selections on the CD30-positive Hodgkin cell line L540. This strategy allowed the construction of a fully human anti-CD30 scFv (hAK30) with the same binding specificity as moab Ki-4, and in which only the VH(CDR3) and framework 4 sequences are derived from the parental antibody. This scFv may serve as a useful building block for the synthesis and engineering of different fusion proteins, such as scFv coupled to toxins, enzymes, or, in connection with other targeting molecules, as bispecific agents (Huston et al, 1993). It is a promising candidate to use as immunotherapeutic agent for the treatment of CD30-positive malignancies.

MATERIAL AND METHODS

Cell lines

The Hodgkin-derived cell line L540 (Diehl et al, 1981) and the hybridoma cell lines BW702 (Bosslet et al, 1989), Ki-3, Ki-4, Ki-6, Ki-7 (Horn-Lohrens et al, 1995) and BerH2 (Schwarting et al, 1989) were maintained in RPMI 1640 medium (GIBCO-BRL, Rockville, MD, USA) supplemented with 10% (v/v) FCS, 100 µg ml^{-1} streptomycin, 200 units ml^{-1} penicillin and 2 mM L-glutamine (10% FCS-medium). All cells were cultured at 37°C in a 5% CO_2 humidified atmosphere.

Bacterial strains and plasmids

E.coli XL1-Blue (supE44, hsdR17, recA1, endA1, gyr, A46, thi, relA1, lacF′. $proA^+$ B^+ $lacI^q$, lacZ_M15, Tn10(tet^r)] were obtained from Stratagene (La Jolla, CA, USA). *E.coli* TG1 (K12_(lac-pro), supE, thi, hsdD5/F′traD36, $proA^+$ B^+, $lacI^q$, lacZ_M15) and *E.coli* HB2151 (K12_(lac-pro), ara, nal^r, thi/F′, $proA^+$ B^+, $lacI^q$, lacZ_M15) were purchased from Pharmacia (Uppsala, Sweden). The phagemid vector pCANTAB6 (McCafferty et al, 1994) is used for N-terminal fusion of scFv fragments to the minor coat protein p3 of filamentous phage M13 using Sfi I (Nco I)/Not I restriction sites. An amber-stop codon between the scFv-gene and the bacteriophage gene 3 allows the expression of soluble fragment or phage-displayed scFv, in an *E.coli* non-suppressor or suppressor strain, respectively.

Chain shuffling of murine Ki-4 V-genes

The murine Ki-4 scFv was synthesized as described (Klimka et al, 1999). From this scFv, the CDR3-linker-VL-gene fragment was amplified by polymerase chain reaction (PCR) using 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, with the primers VH-FR3-BACK (5′-GAC ACG GCY GTR TAT TAC TGT-3′) and FD-TET-SEQ (5′-TTT GTC GTC TTT CCA GAC GTT AGT-3′) and the proof-reading *Pfu*-polymerase (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Simultaneously, human VH genes lacking the CDR3-FR4 sequence were amplified from the pHEN1-human scFv repertoire made by Marks et al (1991), using the primers pUC-REV (5′-CAG GAA ACA GCT ATG AC-3′) and VH-FR3-FOR (5′-ACA GTA ATA YAC RGC CGT GTC-3′). For PCR assembly of the amplified fragments, 250 ng of each were combined in a 50 µl mixture and cycled seven times (94°C for 1.5 min, 65°C for 1 min and 72°C for 2 min) to join the fragments. The reaction mixture was then amplified for 30 cycles (94°C for 1 min, 55°C for 2 min and 72°C for 2 min) after the addition of the outer PCR primers pUC-REV/FD-TET-SEQ. Assembly products were digested with Sfi I/Not I and ligated into the phagemid vector pCANTAB6. The ligation mix was purified by phenol extraction and ethanol precipitation and dissolved in 20 µl H_2O. The DNA solution was transfected into 100 µl *E.coli* TG1 by electroporation as described elsewhere (Dower et al, 1988). The cells were grown for 1 h in 2×TY medium at 37°C before plating on 2×TY agar medium containing 100 µg ampicillin ml^{-1} and 2% (w/v) glucose (2×TY-Amp-Glu).

Five different selected human VH genes, determined by DNA-fingerprint analysis as described elsewhere (Marks et al, 1991) from 20 CD30-reactive half-human scFvs, were amplified with primers pUC-REV/VH1-FOR-Xho (5′-CCG CCT CCA CCA CTC GAG ACG GTG ACC GTG GTC CC-3′) using Pfu-polymerase, ligated into pCANTAB6 using restriction enzymes Sfi I/Xho I and electroporated into *E.coli* XL1-Blue. After sequencing of the human VH genes, they were cloned into the pHEN1-VL repertoire (Marks et al, 1991) using the restriction sites Sfi I/Xho I and transfected into *E.coli* TG1 by electroporation as described above. After selection, the human anti-CD30 scFv (hAK30) was finally cloned into pCANTAB6 using the restriction enzymes SfiI/NotI for expression as His-tagged protein.

Selection of phage on the Hodgkin-derived cell line L540

The resulting repertoires of transformed bacteria containing the murine Ki-4 VL linked to human VH repertoire in phagemid vector pCANTAB6 or selected human VH-genes linked to the human VL repertoire in pHEN1, were rescued with helper phage M13K07 as described (Marks et al, 1991). The selection procedure is described elsewhere (Klimka et al, 1999). Briefly, 5×10^5 L540 cells were incubated with 1 ml of 1×10^{13} cfu ml^{-1} phage in 2% (w/v) MPBS (2% Marvel skimmed milk powder in PBS) for 1 h at room temperature (RT). After washing the cells ten times with

5 ml 2% MPBS and two times with 5 ml PBS by spinning (300 *g*, 3 min, RT) and resuspending respectively, binding phage were eluted with 50 mM HCl and remaining cell debris was spun down (300 *g*, 5 min, RT) after neutralization with 1 M Tris-Cl, pH 7.4. Phage-containing supernatant (SN) was mixed with 3 ml 2×TY-Glu medium and used to transfect logarithmically growing *E.coli* TG1 cells for 30 min at 37°C before plating on 2×TY-Amp-Glu agar medium.

FACS analysis

Cell binding of phage-displayed scFvs was demonstrated by FACS analysis. 5×10^5 L540 target cells were washed in PBS containing 2% (w/v) skimmed milk powder and 0.05% (w/v) sodium azide (2% MPBS/N_3–) and then incubated for 1 h at 4°C with the respective phage or moabs Ki-3 or Ki-4 in 2% MPBS/N_3– respectively. Bound phage were detected with a sheep anti-fd serum (Pharmacia, Uppsala, Sweden; 0.02% (v/v) in 2% MPBS/N_3–) and FITC-labelled rabbit-anti-sheep IgG (Dianova, Hamburg, Germany; 2% (v/v) in MPBS/N_3–). Bound monoclonal antibodies were detected with FITC-conjugated goat-anti-mouse IgG (Becton & Dickinson, Heidelberg, Germany); cells were analysed on a FACScan (Becton & Dickinson). For competition FACS analysis, approximately 10^{12} cfu of phage displaying scFv were mixed with 50 µl of unpurified supernatant from hybridomas secreting moab Ki-3 or moab Ki-4, respectively, resulting in a phage vs moab ratio of approximately 1/1. The mixtures were incubated with the target cells and bound phage were subsequently detected as described.

Sequencing

The scFv-genes were sequenced by the dideoxy chain termination method (Sanger et al, 1977) using Dye-Terminator mix (Perkin Elmer, Norwalk, CO, USA) and the oligonucleotides FD-TET-SEQ and pUC-REV. Products of the sequencing reaction were analysed on a semi-automated ABI Prism sequencer (Perkin Elmer). The nucleic acid sequences of the V regions were compared to the Kabat database of V genes (Kabat and Wu, 1991) and Sanger Centre database (http://www.sanger.ac.uk) to determine the V-gene family and germline V-gene segments.

Purification of recombinant, human, soluble CD30-His

Cloning of the extracellular part of human CD30 receptor fused to a His_6-tag into the eukaryotic expression vector pcDNA3 (Invitrogen, Groningen, The Netherlands) is described elsewhere (Barth et al, 2000). 250 ml supernatant of COS-1 cells transfected with sCD30-His-pcDNA3 plasmid was collected, filtered and incubated with 2 ml Talon™ resin (Clontech, Heidelberg, Germany) for 2 h at 4°C for IMAC purification. The resin was subsequently washed with Tris-buffer (20 mM Trisbase, 100 mM NaCl, pH 8.0) and Tris-buffer, 5 mM Imidazol until the $OD_{280\ nm}$ dropped to 0.001. The sCD30-His protein was then eluted with 250 mM Imidazol in Tris-buffer and dialysed against PBS ovn at 4°C.

Purification of scFv

E.coli HB2151, harbouring the respective scFv genes in pCANTAB6 were used to inoculate 750 ml of 2×TY medium containing 100 µg ml^{-1} ampicillin and 0.1% (w/v) glucose. The culture was grown at 37°C to an $OD_{600\ nm}$ of 0.9 and then supplemented with 1 mM isopropylthio-β-D-galactoside (IPTG) for induction of soluble scFv expression. After 4 h of induction at 30°C, the cells were pelleted and resuspended in 8 ml ice-cold TES (200 mMTris-HCl; 0.5 mM EDTA; 500 mM sucrose). After incubation for 5 min on ice, 8.8 ml of TES/H_2O (1:3) were added and the bacterial suspension was incubated on ice for an additional 20 min. Bacteria were pelleted, SN was collected and the pellet was resuspended in 10 ml TES/15 mM $MgSO_4$ and incubated on ice for 15 min. After centrifugation for 5 min at 300 *g* the supernatants were mixed and centrifuged at 13 000 *g* for 10 min to remove cell debris. The resulting periplasmic fraction was dialysed against Tris-buffer (20 mM Trisbase, 100 mM NaCl, pH 8.0) ovn at 4°C and the scFvs were purified by IMAC using Talon™ resin (Clontech, Palo Alto, USA) as described for the sCD30-His protein.

The human scFv hAK30 was additionally expressed under high-salt stress induction as described elsewhere (Barth et al, 2000). In brief, 2 L bacterial culture were grown at 28°C in TB-medium containing 0.5 mM $ZnCl_2$ and 0.1 M potassium phosphate buffer, pH 7.5 till OD_{600nm} of 1.6. The culture was supplemented with 0.5 M sorbitol, 0.7 M NaCl, 10 mM betain and after 15 min expression was induced by addition of 1 mM IPTG. After overnight growth, bacteria were centrifuged and the pellet was snap-frozen in liquid nitrogen and resuspended in 75 mM Tris-buffer, pH 8.0 containing 10% glycerol, 300 mM NaCl, 2 mM EDTA, 5 mM DTT and Complete™ protease inhibitor (Boehringer Mannheim, Mannheim, Germany). Proteins were extracted by sonification and centrifugation, desalted by gelchromatography using a desalting column (Pharmacia, Uppsala, Sweden) and scFv was isolated by IMAC using Ni-NTA resin (Qiagen, Hilden, Germany).

Eluted protein was thoroughly dialysed against PBS and visualized by gelfiltration, SDS-PAGE and immunoblotting. The final concentration was determined from a scanned Coomassie-stained SDS-PAGE with BSA-dilutions as standards and performing densitometrical analysis with Multi-Analyst software (Bio-Rad, Munich, Germany).

Determination of relative binding affinities of anti-CD30 antibodies

To determine the relative binding affinities of the anti-CD30 antibodies, purified recombinant sCD30-His (70 nM) was incubated for 1 h at RT in duplicates, with dilution series of the respective purified scFvs, the Ki-4 Fab fragment prepared as described elsewhere (Smith, 1993), or the moab Ki-4, respectively. Unbound sCD30-His antigen was detected in a CD30 (Ki-1 antigen)-ELISA kit (DAKO, Glostrup, Denmark) where the coated anti-CD30 antibody BerH2 binds to the same CD30-epitope as the investigated antibodies. sCD30-His captured by BerH2 was detected by peroxidase-conjugated anti-CD30 antibody Ki-1, which binds to a different epitope. The ELISA was performed according to the manufacturer's instructions and extinction at 450 nm was measured. The antibody concentration at which the OD_{450} dropped to 50% of maximum extinction was taken as the apparent K_d.

Measurement of shed sCD30 receptor

2×10^5 L540 cells were washed three times with 10 ml fresh 10% FCS medium and incubated with 1/10 diluted supernatants of

Table 1 Selection of half-human (A) and human (B) anti-CD30 phage antibodies on Hodgkin cell line L540.

Phage clones	Input titre (cfu)	Output titre (cfu)	Ratio (output/input)	Frequency of positive clones in whole-cell ELISA[a]
A				
Before selection	–	–	–	0 of 94 (0%)
1st round of selection	4×10^{13}	2×10^{7}	5×10^{-7}	47 of 94 (50%)
2nd round of selection	2×10^{12}	4×10^{9}	2×10^{-3}	75 of 94 (80%)
B				
Before selection	–	–	–	n.d.
1st round of selection	2×10^{13}	2×10^{6}	1×10^{-7}	n.d.
2nd round of selection	4×10^{13}	1×10^{7}	2.5×10^{-7}	0 of 94 (0%)
3rd round of selection	6×10^{13}	5×10^{8}	8.3×10^{-6}	7 of 93 (8%)

[a]Clones have been stated as positive if $OD_{490\ nm}$ was three times higher than background; cfu, colony forming unit; n.d., not determined.

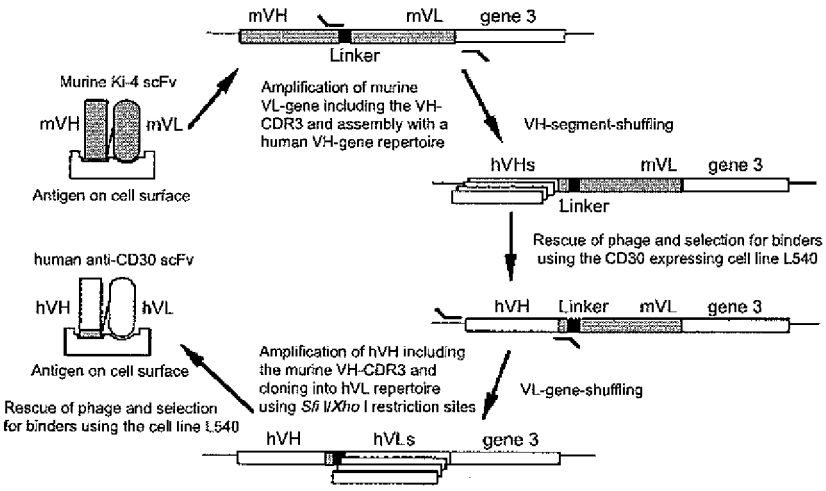

Figure 1 Schematic drawing of the chain-shuffling procedure used for the guided selection of the human anti-CD30 scFv hAK30. The gene 3 encodes the phage minor coat protein p3 and is part of the phagmid vector pCANTAB6.

different anti-CD30 hybridomas or approx. 0.5 μg ml^{-1} purified Ki-4 Fab-fragment, mKi-4 scFv, A12 scFv or hAK30 scFv in 1 ml 10% FCS medium, respectively, to ensure an excess of antibody against the CD30 receptor on the cell surfaces. After 2 h, the cells were washed three times in 10 ml 10% FCS medium by centrifugation (300 *g*) and resuspension to remove unbound antibodies, before the cells were incubated for further 24 h. 100 μl of cell-free supernatants were checked for the level of shed extracellular CD30 receptor using the CD30 (Ki-1 antigen)-ELISA kit (DAKO, Glostrup, Denmark). Relative OD_{450} extinction was determined and compared to the sCD30-level of cells incubated with hybridoma supernatant of an anti-GD2 antibody (BW702) as control.

RESULTS

Cloning of V genes and selection of the half-human and human anti-CD30 scFv

To retrieve a fully human anti-CD30 scFv, the strategy of 'guided selection' (Figure 1) was followed using a recently-cloned murine anti-CD30 scFv (mKi-4 scFv) as guiding molecule. First, the CDR3-linker-VL gene fragment of the murine anti-CD30 Ki-4 scFv was combined with a repertoire of CDR3-truncated human VH genes taken from a repertoire of 1.8×10^{8} human scFv clones (Marks et al, 1991). Phage displaying these combinatorial scFvs were selected for binding to the CD30-positive cell line L540. As documented in Table 1A, two rounds of selection and amplification were sufficient to enrich for CD30-binding, half-human scFv bearing phage (human VH-murine VL) up to 80%, as determined in a whole-cell ELISA using L540 cells. DNA-fingerprint analysis of 12 individual clones with the restriction enzyme BstN I revealed five different patterns in DNA-gel electrophoresis (Figure 2A). The scFv-genes of five representative clones were sequenced and the deduced amino-acid sequences were compared with the Kabat database and the Sanger Centre database of human VH-genes to determine their V-gene family and their closest germline match.

As depicted in Table 2, all five V-genes belong to the VH-I family, with two VH-genes showing the highest homology to the VH DP-75 segment. This segment is also the gene with the highest homology towards the murine VH sequence. The deduced amino-acid sequences of the human and murine Ki-4 heavy-chain CDR1 and CDR2 show a homology of 23–50% (Table 3). However, structural analyses, as far as they can be predicted from the amino-acid sequence (Chothia et al, 1989; 1992), revealed that similar classes of canonical structures for the human and the murine VH-genes occurred.

Table 2 Deduced amino acid sequences of selected VH- and VL-genes

Heavy chains:

```
                  1        10        20        30        40        50        60        70        80        90        100       110
                  |        | FR1     |         | CDR1    |  FR2    |  CDR2   |         |  FR3    |         | CDR3    |  FR4    |
mVH Ki-4          QVKLQESGTELAKPGAAVKMSCKASGYTFT DYWMH WVKQRPGQGLEWIG YINPNTAYTDYNQKFKD KATLTADKSSSTAYMQLRSLTSEDSAVYYCAK KTTQTTWGFPF WGQGTTVTVSS
hVH A9            --Q-----A-VK----S--L---------- NHFI- --R-A-------M- T---SSGS-T-A-R-QG RV-M-R-T-TR-V--E-SR--HD-T------- ---------- ----------
hVH A3            --Q--Q--A-VK----S--V---------- S-GIN -LR-A-------M- G-I-IPGTAN-T-R-Q- RL-I---D-T--S--E-SD-----T------- ---------- ----------
hVH A4            --Q-VQ--A-VK---SS--V------G--S SSSIS --R-A-------M- G-I-SFGTAN-A-R-QG RV-I---E-T-----E-S--R---T-----T- ---------- ----------
hVH A2            --Q-VQ--A-VK--R-S--V---T------- G-Y-- --R-A----F--M- W-D--GGA-T-A---QG RL---R-T-IN----D-SR---D-T------- ---------- ----------
hVH A12(hAK30)    --Q--Q--A-VK---SS--V---T------- G-Y-- --R-A----F--M- W-D--SGA-T-A---QG RLI-SR-T-IN----E--R---D-T------- ---------- ----------
                                    [ H1 ]                    [ H2 ]
```

Light chains:

```
                  1        10        20        30        40        50        60        70        80        90        100
                  |        | FR1     |         | CDR1    |  FR2    |  CDR2   |         |  FR3    |         | CDR3    |  FR4
mVL Ki-4          DIVLTQSPKSMAMSVGERVTLSC KASENVDSFVS WYQQKPGQSPKLLIY GASNRYT GVPDRFAGSGSGRDFTLTISSVQAEDLADYHC GQNYRYPLT FGAGTKLEIK
hVL hAK30hVH      ---M----STLSA---D---IT- R--QG-YQWLA -------KA-N---- K--HL-M ---S--S-----T--------L-PD-F-T-Y- Q-LNS----- --G---V---
                                    [ L1 ]                    [ L2 ]          [ L3                    ]
```

Amino acid sequences of selected VH and VL fragments are aligned towards parental murine Ki-4 fragments. Different amino acids are indicated. Numbering is according to Kabat and Wu (1997). Definition of CDR-loops (H1, H2, L1, L2, L3) is according to Chothia et al (1989; 1992); FR,. framework region; CDR, complementarity determining region.

Table 3 V-gene classifications, structural predictions of the CDR-loops and homologies of V-genes involved in the chain-shuffling procedure

V-genes	V-gene family[a]	Predicted canonical structure of CDR loops[b] H/L 1	H/L 2	H/L 3	Human germline gene with closest deduced protein sequence[c]	Amino-acid sequence homology (%) of CDRs towards murine Ki-4
mVH-Ki-4	Mo-VH VII	1	2	n.a.	VH DP-75	100
hVH A9	Hu-VH1	1	2/3	n.a.	VH hv1f10t	36
hVH A3	Hu-VH1	1	2	n.a.	VH VHGL-1.8	32
hVH A4	Hu-VH1	1	2	n.a.	VH DP-10	23
hVH E2	Hu-VH1	1	2/3	n.a.	VH DP-75	50
hVH A12 (hAK30)	Hu-VH1	1	2/3	n.a.	VH DP-75	50
mVL-Ki-4	Mo-VκXXI	2	1	1	Vκ DPK-24	100
hVL-4 (hAK30)	Hu-Vκ1	2	1	1	VκL12a+	41

[a]V-gene families assigned to Kabat database (http://immuno.bme.nwu.edu/famgroup.html); [b]Canonical structures were determined according to Chothia et al (1989; 1992); [c]germline genes assigned to Sanger Centre database (http://www.sanger.ac.uk); n.a., not applicable

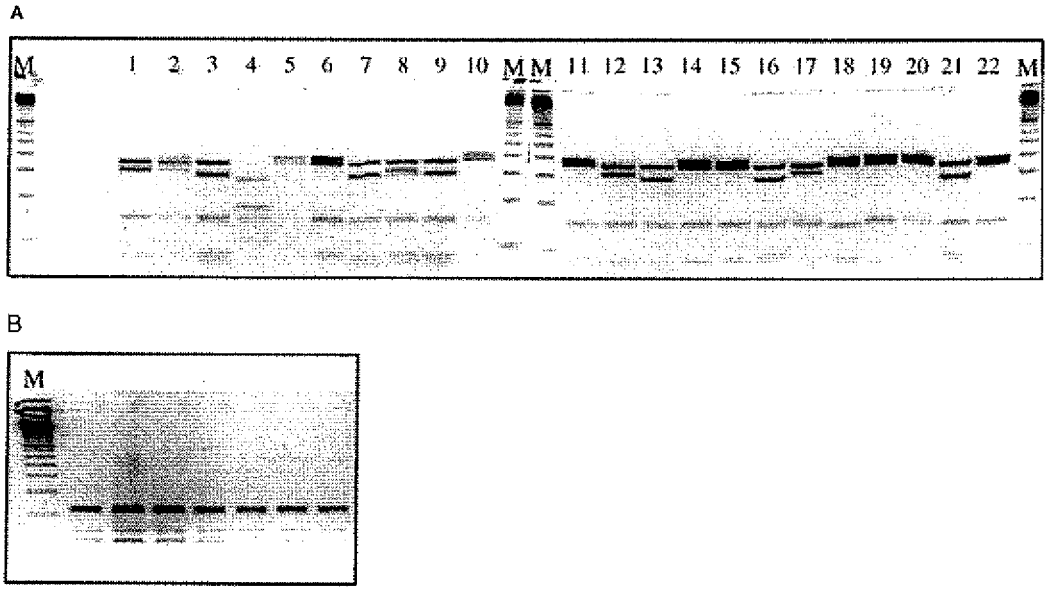

Figure 2 Bst NI fingerprint-analysis of positive scFv clones determined by whole-cell ELISA using cell line L540. The scFv inserts were PCR-amplified from individual colonies using vector-based primers according to Marks et al (1991). The products were digested with Bst NI and analysed on agarose gels. M, 100 bp molecular weight marker. (A) Digests from colonies with half-human scFvs after 1 round of selection (lanes 1 to 10) and 2 rounds of selection (lanes 11 to 22). (B) Lanes 1 to 7 are digest, from colonies with human scFvs after 3 rounds of selection.

The five selected human VH-genes were PCR-amplified and cloned into the phagemid vector pCANTAB6. After sequencing the VH-genes once more, they were pooled and cloned into a 4.5×10^6 member human (h)VL phage antibody library in pHEN-1 (Marks et al, 1991) resulting in a combinatorial library of 8×10^6 individual clones. Three rounds of phage selection and amplification were performed using the Hodgkin-derived cell line L540, which resulted in 8% binders in a whole-cell ELISA (Table 1B). DNA-fingerprint analysis showed that all positive clones contain the same human VH- (from half-human clone A12) and human VL- gene (Figure 2B), which was subsequently confirmed by sequencing two of these clones. The deduced amino-acid sequence of the selected human VL-gene (Table 2) shows that it possesses a 41%-homology to the parental murine VL-gene in the CDR regions and retains similar structural elements (Table 3). The DNA-sequence of the final human anti-CD30 scFv hAK30 was submitted to GenBank (accession number AF117956).

Binding properties of the half-human and human anti-CD30 scFvs

To verify binding specificity of the scFvs against the CD30-epitope recognized by the monoclonal Ki-4 antibody, competition experiments were performed and evaluated by FACS analysis. As shown in Figure 3, binding of the selected scFvs displayed on phage was partially but specifically blocked by the parental Ki-4 moab, but not by the monoclonal Ki-3 antibody, which recognizes a different epitope on the CD30 antigen (shown for mKi-4 scFv, h/mA12 scFv, and human hAK30 scFv). The scF-v-genes were subsequently expressed in *E. coli* non-suppressor strain HB2151

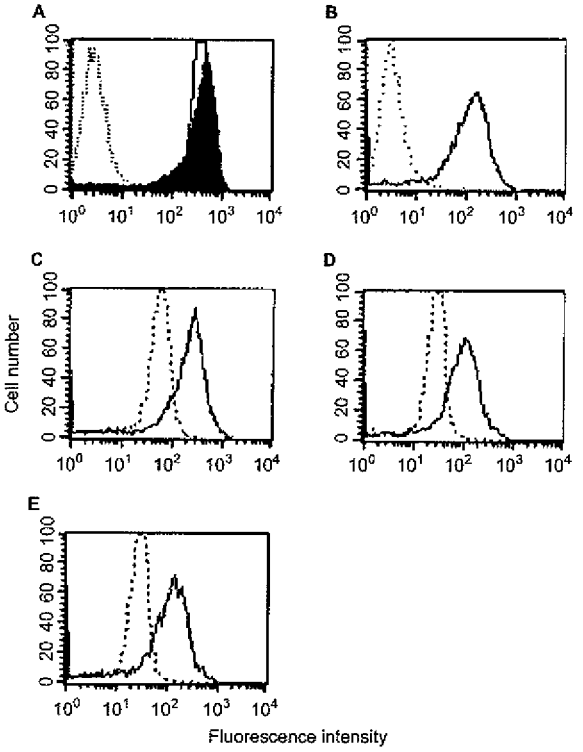

Figure 3 Histograms of FACS-analysis for determination of the CD30-epitope specificity of the selected anti-CD30 scFvs. (**A**) L540-cells were incubated with anti-CD30 Ki-3- (filled area), Ki-4 (unfilled area) hybridoma-supernatant or PBS (dotted line) and binding was detected by FITC-conjugated goat-anti-mouse IgG antibody. (**B**) L540 cells were incubated with phage displaying no scFv (dotted line) or phage displaying half-human scFv A12 (black line). L540 cells were incubated with phage-antibodies mKi-4 (**C**), h/mA12 (**D**) or hAK30 (**E**) and additionally with anti-CD30 moab Ki-3- (black line) or Ki-4- (dotted line) hybridoma-supernatant, respectively. Binding of phage-antibodies was subsequently detected using sheep-anti-M13-serum and FITC-conjugated rabbit-anti-sheep-IgG antibody.

and purified by IMAC. The typical yield of purified scFv was approximately 150 μg l^{-1} bacterial culture performing a standard periplasmic extraction, or twice as much using a modified protocol (see Material and methods).

The relative binding affinities were determined by ELISA using a defined concentration of purified sCD30-His protein as antigen and dilution series of the indicated anti-CD30 antibodies (Figure 4). Purified scFv antibodies consisted of at least 95% monomeric molecules demonstrated by gelfiltration (data not shown). Antigen and antibodies were incubated in solution and unbound antigen was subsequently quantified with a coated anti-CD30 antibody, recognizing the same epitope as the investigated antibodies. The antibody concentration at which 50% of the antigen was bound at equilibrium was taken as the apparent K_d. The relative affinities of the moab Ki-4 Fab-fragment, the murine Ki-4 scFv and the half-human anti-CD30 scFv A12 are approximately 10-fold higher than the affinity of the human scFv hAK30 (Table 4), but 10-fold lower than the whole, bivalent monoclonal antibody Ki-4.

Shedding-inhibition of the extracellular part of the CD30 receptor

To investigate the influence of our recombinant anti-CD30 antibodies on the shedding of the extracellular part of the CD30 receptor, L540 cells were incubated with supernatants of different anti-CD30 hybridomas or purified recombinant anti-CD30 scFvs, respectively. After 2 h, the cells were thoroughly washed with

Table 4 Apparent affinities of anti-CD30 antibodies

Anti-CD30 antibody	K_d (M)
moab Ki-4	4×10^{-10}
Ki-4 Fab-fragment	5×10^{-9}
mKi-4 scFv	3×10^{-9}
h/mA12 scFv	7×10^{-9}
hAK30 scFv	3×10^{-8}

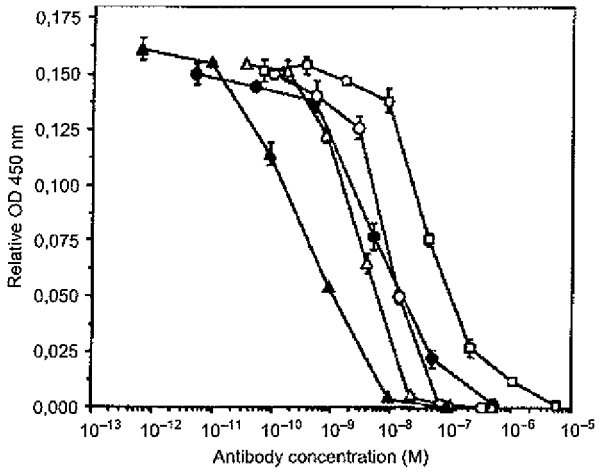

Figure 4 Apparent binding affinities of anti-CD30 antibodies using recombinant sCD30-His antigen. Dilution series of anti-CD30 antibodies moab Ki-4 –▲–, Ki-4 Fab-fragment –●–, mKi-4 scFv –△–, h/mA12 scFv –○– and hAK30 scFv –□– were incubated with recombinant sCD30-His protein and unbound antigen was detected by CD30-ELISA (DAKO).

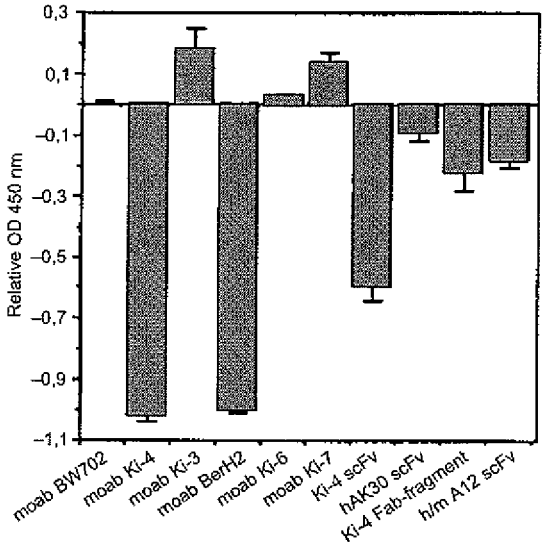

Figure 5 Influence of anti-CD30 antibodies towards naturally occuring CD30 receptor cleavage from Hodgkin-derived L540 cell line. Cells were incubated for 2 h with indicated antibodies and shed CD30 receptor was detected by CD30-ELISA (DAKO). Value of relative OD_{450} of the control, using an irrelevant anti-GD2 antibody (BW702), was set as zero baseline.

medium to deplete unbound antibodies. After another 24 h of incubation, supernatants were checked for sCD30-level in a CD30 (Ki-1 Antigen) ELISA kit (DAKO) in duplicates. Figure 5 shows that, as was described by Horn-Lohrens et al (1995), Ki-4 and BerH2 strongly inhibit the shedding of the extracellular part of CD30 receptor (sCD30), whereas Ki-3, Ki-6 and Ki-7 increased the sCD30 level to different extents. The recombinant Ki-4-derived anti-CD30 antibodies and the monovalent moab Ki-4 Fab fragment exhibit a comparable inhibition of the cleavage of CD30 receptor from L540 cells, although they are not as potent as the bivalent moabs Ki-4 and BerH2.

DISCUSSION

In this paper, we report the cloning of a human anti-CD30 scFv by guided selection from the murine anti-CD30 scFv Ki-4 using the phage display technology. In contrast to other described procedures (Jespers et al, 1994; Figini et al, 1994), we retained the VH-CDR3 region of the parental murine scFv in this guided selection. This particular region is not only known for its significant importance in determining the binding specificity of an antibody, it is also a highly variable region in every antibody, which makes it less likely to be a major immunogenic part of the molecule. We believe that this region was important in retaining the CD30-epitope specificity of the parental antibody in the human scFv. Watzka et al (1998) describe the humanization through chain-shuffling of an anti-human interferon γ receptor I antibody without retaining the VH-CDR3 region of the murine antibody. The resulting fully-human Fab-antibody was antigen specific, but differed in epitope specificity from the parental hybridoma, thereby underlining the importance of the VH-CDR3. This importance of the VH-CDR3 for epitope specificity has also recently been reported by Beiboer et al (2000), thereby confirming our findings.

Selection of phage libraries in this study was performed on a Hodgkin-derived cell line which is known for its high surface expression of CD30 receptor (10^6 receptor molecules per cell), rather than by panning on recombinant CD30 antigen. CD30 is part of the TNF receptor family and many ligands and receptors are known to trimerize. It may therefore not be straightforward to retain natural epitopes on recombinant versions of such cell-surface molecules. Therefore, cell panning on CD30-positive cells is a good and valid alternative. Indeed, using recombinant CD30 protein for panning of several human scFv phage repertoires, to date other groups were unsuccessful in retrieving functional anti-CD30 antibodies.

The selection on cells and therefore native CD30 receptor resulted in five different human VH genes with homology in the CDR1 and CDR2 regions between 23–50%, compared with the parental murine Ki-4 heavy chain. This is similar to what was found in the group of Watzka et al (1998) for their selected anti-human interferon γ receptor antibody (45%), and higher than described in the study of Figini et al (1998), in which an anti-ovarian carcinoma Fab fragment was humanized by guided selection. However, more important than sequence homology might be the length of the CDR regions and the canonical structure of the CDR-loops defined by Chothia et al (1989; 1992). This was striking in the case described by Figini et al (1994) retrieving a human anti-phOx Fab fragment by guided selection which shared these structural elements with the parental mouse antibody. In our study, the selected human VH gene with the highest sequence homology towards mKi-4 VH, but probably slightly different canonical folds, is the one of the half-human anti-CD30 scFv A12. This clone was also predominant after the selection (Figure 2A). Additionally, this human VH gene was selected out of five others after shuffling with the human VL-repertoire. The human VL gene of the finally selected human anti-CD30 scFv has the same length and predicted canonical structure of the CDR-regions as the mKi-4 VL gene, and a 41% homology of the deduced amino-acid sequences concerning these regions. This follows the prediction made by Jespers et al (1994) that there may be a strong preference for retaining V-gene segments with identical canonical folds in guided selection procedures.

Expression of the human anti-CD30 scFv hAK30 as soluble fragment revealed a 10-fold lower apparent K_d for the hAK30 scFv compared with the mKi-4 scFv (Table 4). A loss in affinity after a guided selection procedure has also been reported by other groups (Figini et al, 1998). However, the hAK30 scFv reveals an affinity in the nanomolar range and is therefore expected to be adequate for use as targeting moiety in recombinant immunotherapeutics, in particular when re-formatted as bivalent molecule (Tai et al, 1995). The relative affinity of the monovalent Ki-4 Fab-fragment (5×10^{-9} M) is comparable to the value measured for the mKi-4 scFv and underlines the successful cloning of the functional V-genes from the hybridoma Ki-4. The higher affinity of the bivalent moab Ki-4 (3.7×10^{-10} M) most probably is caused by an avidity effect in the assay.

The monoclonal antibody Ki-4, as well as the moab BerH2, significantly inhibit the naturally occurring shedding of the extracellular part of the CD30 receptor, as demonstrated by Horn-Lohrens et al (1995). Since this is a desired property for an anti-CD30 antibody as part of an immunotherapeutic agent, we were especially interested in retaining the epitope-specificity of the moab Ki-4 in our human anti-CD30 antibody. As shown in Figure 3, the epitope-specificity was retained for the murine and the human scFv. Although the binding of phage antibodies was not completely inhibited by moab Ki-4, which might be due to higher avidity effects of phage (displaying up to five scFv molecules on their surface), competition for binding was not observed by addition of moab Ki-3. The anti-sCD30-shedding property was retained as well, although it was significantly weaker for the monovalent anti-CD30 molecules, which correlates with their apparent binding affinities (Figure 5). A bivalent scFv, like a diabody, may even be as potent as the bivalent moab Ki-4 regarding the CD30-shedding inhibition. Whether the human anti-CD30 scFv hAK30 will be as potent as the murine Ki-4 scFv as part of an anti-CD30 immunotherapeutic agent (Klimka et al, 1999) has to be further analysed, e.g. by fusing it to a human-derived toxin gene (Newton et al, 1996) in order to get a fully human, recombinant immunotoxin.

In summary, we have been able to derive a functional human anti-CD30 scFv (hAK30) from the murine anti-CD30 scFv Ki-4 by guided selection using human V-gene repertoires and phage display technology. The hAK30 scFv retains the epitope specificity of its murine counterpart and inhibits the shedding of the CD30 receptor from the cell surface.

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